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Award Number:

W81XWH-04-1-0209

TITLE:

Notch Signaling and Schwann Cell Transformation: Development
of a Model System and Application to Human MPNSTs

PRINCIPAL INVESTIGATOR:

Tom Kadesch, Ph.D.

CONTRACTING ORGANIZATION:

University of Pennsylvania
Office of Research Services
P-221 Franklin Bldg.
3451 Walnut Street
Philadelphia, PA 19104-6205

REPORT DATE:

September 2008

TYPE OF REPORT:

Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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| REPORT DOCUMENTATION PAGE | | | | Form Approved OMB No. 0704-0188 | |
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| 1. REPORT DATE (DD-MM-YYYY) 01-09-2008 | | 2. REPORT TYPE Final | | 3. DATES COVERED (From - To) 1 MAR 2004 - 31 AUG 2008 | |
| 4. TITLE AND SUBTITLE Notch signaling in Schwann cell transformation: Development of a model system and application to human MPNSTs. | | | | 5a. CONTRACT NUMBER | |
| | | | | 5b. GRANT NUMBER W81XWH-04-1-0209 | |
| | | | | 5c. PROGRAM ELEMENT NUMBER | |
| 6. AUTHOR(S) Tom Kadesch, Ph.D. E-Mail: kadesch@mail.med.upenn.edu | | | | 5d. PROJECT NUMBER | |
| | | | | 5e. TASK NUMBER | |
| | | | | 5f. WORK UNIT NUMBER | |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pennsylvania Philadelphia, PA 19104-6145 | | | | 8. PERFORMING ORGANIZATION REPORT NUMBER | |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012 | | | | 10. SPONSOR/MONITOR'S ACRONYM(S) | |
| | | | | 11. SPONSOR/MONITOR'S REPORT NUMBER(S) | |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited | | | | | |
| 13. SUPPLEMENTARY NOTES | | | | | |
| 14. ABSTRACT This is a final report that presents data obtained during the grant's duration of funding. The grant addresses the potential role of Notch signaling in the malignant transformation of neurofibromas to MPNSTs in patients with NF1. Our previous work has shown that constitutive expression of Notch can transform rat Schwann cells and that at least on MPNST-derived human Schwann cell line (of three examined) signals via Notch. This report includes results pertaining to the Tasks of the Statement of Work, including our observation that NICD is NOT sufficient to transform primary Schwann cells. This led to a new Task that addressed this discrepancy and results that showed that NICD provides a Raslike activity for the transformation of Schwann cells. | | | | | |
| 15. SUBJECT TERMS NF1, neurofibroma, MPNST, Notch, Schwann, transformation | | | | | |
| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 19 | 19b. TELEPHONE NUMBER (include area code) |

Table of Contents

| | |
|---|-----------|
| Introduction | 4 |
| Body | 4 |
| Key Research Accomplishments | 17 |
| Reportable Outcomes..... | 18 |
| Conclusions | 18 |
| References | 19 |
| Personnel Supported by Grant..... | 19 |

INTRODUCTION

The goals of the project were to gain insights into the mechanism by which Notch (in the form of its constitutive form, NICD) transforms rat Schwann cells, and to establish the relationship, if any, between Notch signaling and human MPNSTs.

Notch comprises a family of transmembrane receptors whose interaction with ligand leads to proteolytic cleavages that liberate the Notch intracellular domain, NICD, from the plasma membrane. NICD then enters the nucleus where it activates transcription. Notch's role in several cancers is well established, most notably in T-ALL where a rare chromosomal translocation interrupts the Notch1 gene, resulting in the constitutive expression of NICD. Work has shown that nearly 50% of T-ALLs carry more subtle mutations in the Notch1 gene(1). We have shown that forced expression of NICD can transform rat Schwann cells and that one of our three MPNST cell lines expresses detectable NICD(2). We therefore proposed that Notch signaling may contribute to the malignant transformation of a subset of neurofibromas in NF1 patients.

BODY

- Task 1. Determine the cause of NICD expression in MPNST cells.
- Determine genomic organization of the Notch genes and expression of Notch ligands in sNF96.2 cells.

We reasoned that the expression of NICD in sNF96.2 cells may be due either to chromosomal rearrangements involving any one of the four the Notch genes (leading to ligand-independent activation) or to over-expression of any one of the five different Notch ligands. Our data have ruled out rearrangements of the Notch1 gene (Southern blots; data not shown) and most likely the other Notch genes as well. Although tentative, the latter conclusion stems from a) the relatively low level of NICD expression in sNF96.2 cells (compared to a T cell line carrying a translocation of the Notch1 gene; data not shown) and b) the dramatic increase in NICD that results when sNF96.2 cells are depleted of calcium (data not shown). Removing calcium activates endogenous Notch genes by shedding their extracellular domains and thus the observation indicates that the endogenous Notch proteins in sNF96.2 cells are not constitutively active. As for ligands, we have determined that Jagged1 is not highly expressed in sNF96.2 cells (data not shown). Expression of the other four ligands has yet to be evaluated.

Conclusions. We are relatively confident that the NICD expression in sNF96.2 MPNST cells is not due to a grossly rearranged Notch gene. However, work from Jon Aster and Thomas Look has identified subtle Notch mutations that can activate signaling and play a participatory role in hematopoietic malignancies. Such mutations have not yet been looked for in sNF96.2 cells. We have yet to fully evaluate the potential role of ligand-induced signaling.

- Task 2. Identify the pathways and proteins that collaborate with Notch to induce transformation of rat Schwann cells.
- Examine involvement of Ras-MAPK, PI-3 Kinase, AKT, Jak/Stat and NF- κ B.
 - Evaluate status of p16Ink4a, E2Fs, and Sox10.
 - Determine effect of Notch ligands on transformation-associated phenotypes.

Growth properties of NICD-transduced rat Schwann cells. One of the questions we addressed concerns *why* NICD-transduced Schwann cells are transformed. We considered two

possibilities: 1) they grow faster and/or 2) they do not undergo contact inhibition. Growth curves comparing parental and NICD-transduced cells (Figure 1) showed conclusively that NICD is not mitogenic (indeed, NICD-transduced cells grow somewhat slower), but does allow cells to grow well past confluence, to densities much higher than those observed with parental cells. As with the parental cells, NICD-transduced cells remain forskolin dependent (data not shown), arguing further that NICD does not act as a mitogen.

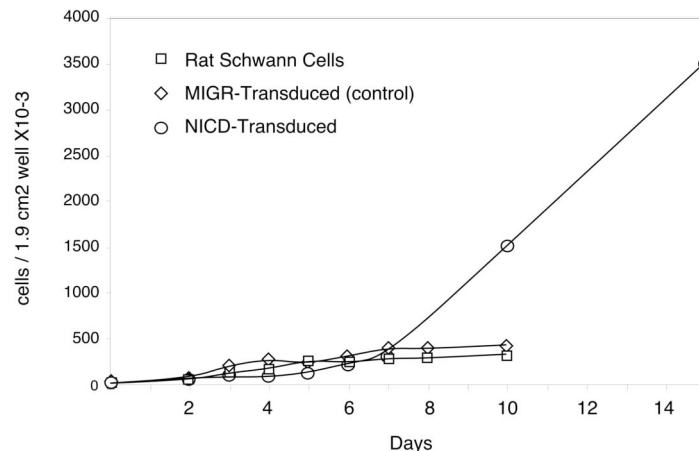


Figure 1. NICD-transduced rat Schwann cells are not contact inhibited. Primary rat Schwann cells, MIGR (control vector) transduced and NICD transduced Schwann cells were plated at day zero and cell counts were determined at the indicated times.

The effect of Notch ligands on rat Schwann cells: the CyclinD1 paradox. It is generally believed that forced expression of NICD leads to much higher levels of Notch signaling than does ligand-mediated activation of endogenous Notch receptors. It was therefore of interest to determine if Notch ligands were able to mimic any of the effects of NICD. We have established techniques in the lab to activate endogenous Notch receptors through ligands (Jagged1 and Delta4) immobilized on tissue culture plates(3). When rat Schwann cells were grown on such plates, they did NOT become morphologically transformed (by appearance), nor did they escape contact inhibition (data not shown). This suggests that the ability of NICD to transform rat Schwann cells a consequence of high level signaling.

We have reported that NICD-transduced Schwann cells express abundant CyclinD1(2). By contrast, we have observed that Notch signaling leads to the *down*-regulation of CyclinD1 in many other cell types, including epithelial cells, fibroblasts and myoblasts (data not shown). Since these latter cell types are not transformed by NICD, we *hypothesized that the response of CyclinD1 to Notch may be symptomatic of a particular cell type's ability to be transformed, those cells that down-regulate CyclinD1 being refractory*. Accordingly, we examined the response of CyclinD1 to ligand-induced Notch signaling in rat Schwann cells. As shown below (Figure 2), rat Schwann cells grown on control plates (Fc) expressed a readily detectable level of CyclinD1 while those grown in the presence of either Delta4 (D) or Jagged1 (J) expressed much less protein. By contrast, rat Schwann cells transduced with NICD expressed an elevated level of CyclinD1 that was not affected by either Delta4 or Jagged1. These data contradict our hypothesis and show that while the inhibitory effect of Notch signaling on CyclinD1 is not symptomatic of a cell type's resistance to transformation by NICD, they do show that once transformed by NICD, CyclinD1 expression is refractory.

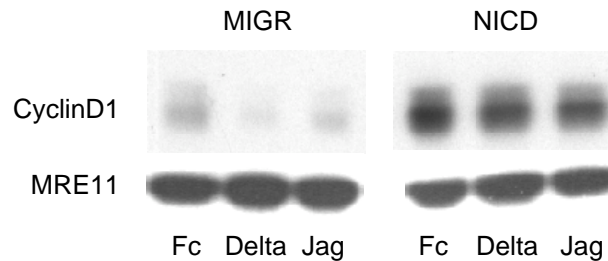


Figure 2. Notch signaling down-regulates CyclinD1 in Rat Schwann cells. MIGR (control) or NICD transduced rat Schwann cells were grown on control plates (Fc) or plates coated with either Delta4 (Delta) or Jagged1 (Jag) and harvested after 72 hours. Levels of endogenous CyclinD1 and MRE11 (control) were assessed by Western blot.

Our experiments have shown that the negative effect of Notch signaling on CyclinD1 is post-transcriptional; that is, Notch does not significantly affect the level of CyclinD1 mRNA (data not shown). The stability of CyclinD1 protein is affected by at least two pathways. One involves GSK3-mediated protein destabilization through phosphorylation of Thr286(4). The other involves an APC-related destabilization through a Destruction Box in CyclinD1's N-terminus(5). To better understand the mechanism by which Notch signaling lowers the level of CyclinD1 protein we introduced into NIH 3T3 cells retroviruses that express various Flag-tagged CyclinD1s, either wild type or mutant. The L32A mutant lacks the Destruction Box while the T286A mutant destroys the primary GSK3 phosphorylation site. As shown below (Figure 3), only the T286A mutant was refractory to the effect of Notch signaling. Although this result implicates GSK3 in mediating the effect of Notch on CyclinD1 expression, we have not observed a difference in either GSK3 levels or GSK3 phosphorylation (phospho-GSK3 has reduced activity) in cells exposed to Notch ligands (data not shown).

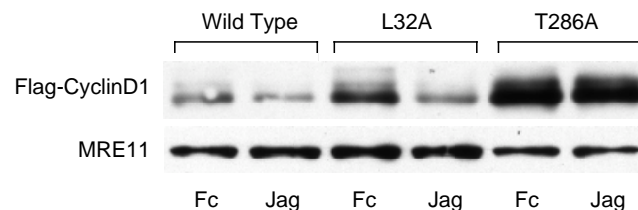


Figure 3. The CyclinD1 T286A mutant is resistant to the inhibitory effects of Notch signaling. Retroviruses expressing Flag-tagged Wild Type, L32A or T286A CyclinD1 were introduced into NIH 3T3 cells and then the cells were grown on plates coated with Fc (control) or Jagged1 (Jag) for 24 hours. CyclinD1 levels were assessed by Western blot using an anti-Flag antibody and an MRE11 antibody as control.

We next examined the response of CyclinD1 to Notch signaling in our three MPNST cell lines. We reasoned that if Notch signaling is largely responsible for transformation, particularly in the sNF96.2 cells which express NICD(2), then CyclinD1 in those cells should not be responsive to increased Notch signaling, similar to what we observed for NICD-transformed rat Schwann cells (Figure 2). As shown below (Figure 4), all three MPNST cell lines in addition to our NF cell line down-regulated CyclinD1 when grown on Notch ligand for either 48 hours (upper panel) or 72 hours (lower panel).

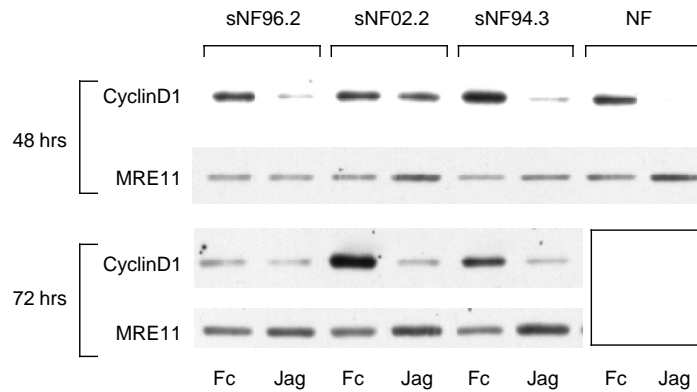


Figure 4. MPNST cells down-regulate cyclinD1 in response to Notch signaling. MPNST cell lines (sNF96.2, sNF02.2, sNF94.3) and an NF cell lines were grown on control plates (Fc) or plates coated with Jagged1 (Jag) for 48 or 72 hours as indicated. Endogenous CyclinD1 was assessed by Western using an anti-MRE11 as loading control. (Note that the NF cell line was not evaluated at 72 hours.)

Conclusions. If the resistance of CyclinD1 to Notch signaling is a hallmark of Notch-mediated transformation, then we conclude that none of our MPNST cell lines, including sNF96.2, is transformed as a consequence of Notch signaling alone. Furthermore, since NICD-mediated transformation of rat Schwann cells is due to a loss of contact inhibition, future experiments with MPNST cell lines will have to address specifically the role of Notch, if any, in reducing contact inhibition (i.e. their growth in soft agar).

CyclinD1 levels as a function of cell density. Our first experiment was to confirm that CyclinD1 levels were higher in NICD-transformed cells irrespective of cell density. This would rule out artifacts due to the lack of contact inhibition in NICD-transformed cells. As shown in the figure below, CyclinD1 levels were, in fact, higher in NICD-transformed cells irrespective of cell density.

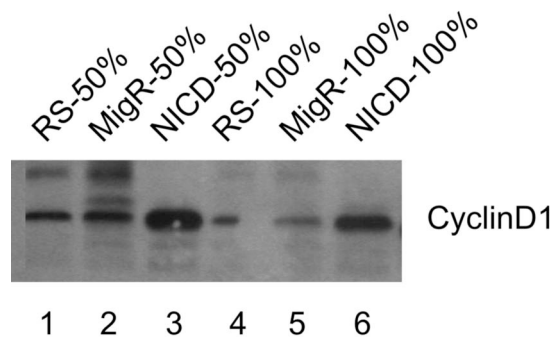


Figure 5. CyclinD1 expression is high in NICD-transformed Schwann cells. Primary rat Schwann cells (RS), and their MigR-transduced and NICD-transduced counterparts were grown to different densities (approximately 50% or 100% confluent as indicated) and CyclinD1 levels were assessed by Western immunoblot.

Cell cycle analyses. Since Cyclin D1 levels are indeed higher in NICD-transformed cells, then these cells should exhibit an altered cell cycle profile, also independent of cell density. We harvested parental (MigR-transformed) and NICD-transformed cells at different densities (roughly 50% confluent and 100% confluent) and carried out cell cycle analyses using propidium iodide (PI) staining and fluorescence activated cell sorting (FACS). The accompanying table shows that NICD-transformed cells had a higher percentage of cells in S-phase at both low density and high density. This argues that the G1-S transition is faster in NICD-transformed cells, a result expected if CyclinD1 levels are higher.

| | Percentage cells in: | | |
|-----------|----------------------|----|----|
| | G1 | G2 | S |
| MigR 50% | 77 | 9 | 14 |
| MigR 100% | 81 | 8 | 11 |
| NICD 50% | 52 | 15 | 33 |
| NICD 100% | 69 | 11 | 20 |

Although we would generally expect populations with a higher percentage of cells in S-phase to be growing faster this case they do not — it appears from the above table that cells transformed with NICD are also more delayed in the G2-phase. Thus, these cells may actually not have an overall shorter cell cycle duration and faster rate of division.

Signaling pathways required for growth in soft agar. Our previous data indicated that NICD-transformed cells, but not parental Schwann cells, could grow in soft agar(2). These results were purely qualitative. We have now repeated these assays, using Ras-transformed NIH 3T3 cells as a control and have included inhibitors of various signaling pathways. It should be pointed out first that we have been able to repeat the soft-agar results (they were performed initially in the lab of our collaborator Dr. Gihan Tennekoon), but that the inherent efficiency is extremely low, being roughly 1 percent that obtained with Ras-transformed NIH 3T3 cells. With regards to the inhibitors, we saw no growth in the presence of Ly, an inhibitor of PI3 Kinase, and very few colonies in the presence of H7, an inhibitor of PKA. Small or no effects were seen in the presence Bis, an inhibitor of PKC, or PD18059, an inhibitor of ERK 1 and 2. We conclude that Notch is either activating or collaborating with the PKA and PI3 Kinase pathways to transform rat Schwann cells.

Effects of Notch ligands and NICD on CyclinD1 transcription. The CyclinD1 promoter has been proposed to be a direct target of NICD(6). Although this is not consistent with our data concerning the down-regulation of CyclinD1 by ligand-induced signaling (see 2005 report), we considered the possibility that *high* concentrations of NICD might be able to transcriptionally activate CyclinD1. Accordingly, we have used quantitative RT-PCR (Q-RT-PCR) to evaluate the effects both of Notch ligands and of NICD on CyclinD1 transcript levels. As shown in the figure below (far right panel), CyclinD1 levels were unchanged when rat Schwann cells were cultured on Fc-Jagged1 and induced slightly (~2.5-fold) in cells transformed with NICD. We conclude 1) that the effect of NICD on the CyclinD1 promoter is dose dependent and 2) that if the CyclinD1 promoter is a direct target of NICD, it is not particularly responsive. Note for comparison the magnitude of NICD-mediated induction of Hes5 or of Hey1.

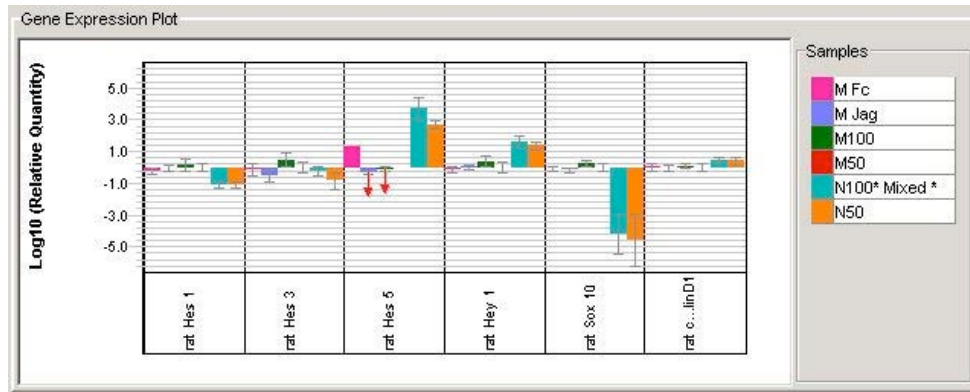


Figure 6. Effects of Notch signaling on the transcript levels of presumptive targets in Schwann cells. Cells were grown under the following conditions, from left to right in each panel: MigR-transduced cells grown a) on Fc-Control (M Fc), b) on Fc-Jagged1 (M Jag), c) to ~50% confluence and d) to ~100% confluence; NICD-transformed cells were grown e) to ~50% confluence and f) to ~100% confluence. RNA samples were analyzed by Q-RT-PCR for expression of the Notch targets Hes1, Hes3, Hes5, and Hey1 and for expression of Sox10. The data represent the averages of three separate experiments.

Growth of NICD-transformed cells in soft agar was completely dependent on signaling through PI3 Kinase (see above). PI3 Kinase is known to activate AKT, which is known to inhibit GSK3, which is known to destabilize Cyclin D1. (In effect, PI3K activity, through AKT, stabilizes CyclinD1 protein). Indeed, some recent reports have shown a correlation between Notch signaling and activation of AKT in T cells(7). Thus, the increase of CyclinD1 protein in NICD-transformed Schwann cells could be due to transcriptional effects as well as to effects on protein stability. Future experiments to address the relationship between NICD and CyclinD1 should a) examine the status of AKT1 and b) determine by ChIP if NICD is actually recruited to the CyclinD1 promoter. (This latter experiment actually falls into the category of Task 4, "Identify primary Notch target genes in rat Schwann cells.")

Expression of additional Notch target genes. The Q-RT-PCR results above also provide data concerning the transcript levels of other Notch target genes. Our initial experiments showed little change in Hes1 protein levels when Schwann cells were transformed with NICD(2) and this correlates with an slight decrease in Hes1 transcript levels (panel 1). Hes3 levels were relatively unchanged (panel 2), while levels of Hes5 transcripts were significantly induced by NICD (panel 3), as expected from higher levels of Hes5 proteins in these cells(2). Levels of Hey1 transcripts were also increased in the presence of NICD (panel 4). Remarkably, the level of Sox10 transcripts were drastically reduced by NICD (panel 5; not that these data are plotted on a log scale). Given the importance of Sox10 in regulating neural crest development and gliogenesis, this result is very exciting and implicates a role for Notch, directly or indirectly through NICD, in regulating Sox10 transcription. Very little is currently known about the Sox10 promoter.

Investigating a possible role for Stat3 in mediating the effects of NICD. It has been shown recently that Notch signaling promotes Jak-Stat signaling in neuronal cells(8). This effect is through the direct binding of either Hes1 or Hes5 to both Jak2 and the DNA binding protein Stat3. Presumably, the Hes proteins facilitate the phosphorylation and activation of Stat3 by

1 Preliminary results show that the status of AKT is not influenced by NICD. However, these experiments have not been repeated.

Jak2. Given that constitutively active Stat3 can be oncogenic(9), and that Hes5 is highly induced in NICD-transformed Schwann cells(2), we felt that it was imperative to investigate a possible connection between NICD and Stat3 in Schwann cells. However, as shown in the representative experiment below, Notch signaling had no such effect in Schwann cells. First, it is interesting to note that rat Schwann cells have relatively high levels of phosphorylated Stat3 to begin with. Second, NICD-transformed cells did not contain higher levels of phosphorylated Stat3 (in fact, they consistently contained slightly lower levels; compare lane 3 to lane 1). Third, growth of cells on the Notch ligand Delta4 did not influence the total amount of phosphorylated Stat3 (compare lane 2 to lane 1). We conclude that if Hes5 does facilitate an interaction between Jak2 and Stat3 in Schwann cells, it is not sufficient to increase the already-high levels of phosphorylated Stat3 and, thus, does not contribute to the transformed phenotype.

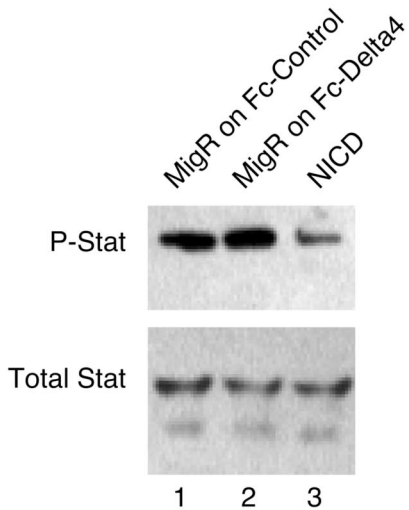


Figure 7. Notch signaling does not influence the level of phosphorylated Stat proteins in rat Schwann cells. Schwann cells were transduced with either a parental retrovirus (MigR) or an NICD-expressing virus. MigR containing cells were cultured on either a control Fc-fusion protein or an Fc-Delta4 fusion protein to initiate Notch signaling. Extracts were made and assessed for expression of total Stat protein or phosphorylated Stat (P-Stat) by Western immunoblot.

Task 4. Identify primary Notch target genes in rat Schwann cells.

- a. Determine the role of Hes-5 in transformation.
- b. Generate, clone and analyze Notch targets using DamID fusion proteins.
- c. Compare gene expression in transformed rat Schwann cells and MPNSTs.

A molecular signature of Schwann cell transformation. We began to use quantitative RT-PCR (Q-RT-PCR) to assess gene expression changes in NICD-transformed Schwann cells. The most notable change involved the dramatic down-regulation of Sox10 mRNA. As shown in Figure 8, we also observed the up-regulation of CyclinD1 and the Notch target genes Hes5, Hey1 and c-Myc. (c-Myc has been identified as a direct target of NICD in T cells; we do not see this in all cell types.)

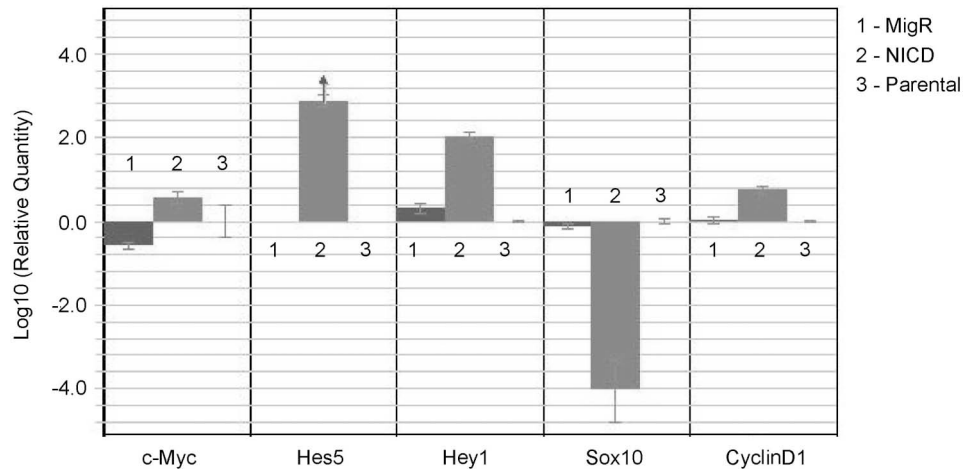


Figure 8. NICD-mediated transformation is accompanied by increased expression of Hes5, Hey1, c-Myc and Cyclin D1 and dramatic inhibition of Sox10 expression. Untransduced cells (3 - Parental), empty virus-transduced (1 - MigR) or NICD-transduced cells (2 - NICD) were evaluated for the expression of the indicated genes using quantitative RT-PCR (Q-RT-PCR).

Constitutive expression of Hes5 or c-Myc is not sufficient for Schwann cell transformation. We sought to determine if either Hes5 or c-Myc was sufficient to induce a transformed phenotype in Schwann cells. Accordingly, we obtained retroviruses that constitutively express either Hes5 or c-Myc and used them to transform primary rat Schwann cells. As shown in Figure 9, constitutive expression of Hes5 did not affect expression of Sox10, Cyclin D1 or c-Myc and so we conclude that Hes5 is unable to mimic the effect of NICD. While constitutive expression of c-Myc was able to enhance the expression of Cyclin D1, it did not down-regulate Sox10 (it actually up regulated Sox10) and it did not result in the loss of contact inhibition (data not shown). We conclude that constitutive expression of c-Myc is also unable to mimic the effects of NICD.

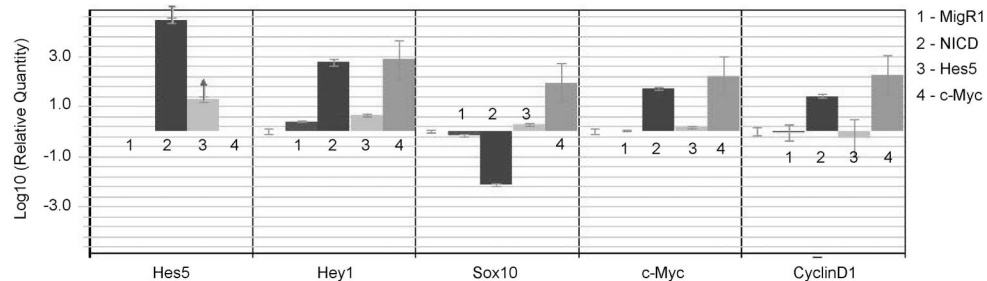


Figure 9. Constitutive expression of Hes5 or c-Myc does not lead to Schwann cell transformation. Schwann cells transduced empty virus (1 - MigR1) or virus expressing NICD (2 - NICD), Hes5 (3 - Hes5) or c-Myc (4 - c-Myc) were evaluated for expression of the indicated genes using Q-RT-PCR.

- Task 3. Determine if Notch-mediated transformation is reversible (Months 6-36).
- Generate and analyze reversibly inducible forms of Notch.
 - Examine effects of Notch inhibitors (γ -Secretase inhibitors, DN-Mastermind).
 - Establish effects of Notch inhibitors on NICD-expressing MPNST cells.

Normal Notch signaling requires three proteolytic events that occur in order within the Notch protein to generate NICD, the active form of the receptor. The first occurs independently of ligand while the second is ligand dependent. The third cleavage is constitutive, but depends on the second having occurred beforehand. The third cleavage is carried out by a multiprotein complex referred to as g-Secretase. g-Secretase inhibitors (GSIs) therefore inhibit signaling by preventing the generation of NICD. An artificial form of Notch that mimics the receptor after the first two cleavages is active since it can be acted on by g-Secretase to generate NICD. This form of Notch, which we refer to as NDE, is inactive in the presence of GSI.

Our approach to determine if Notch signaling is reversible employed the use of NDE. We anticipated first transforming Schwann cells with a retrovirus that expresses NDE and then treating the cells with GSI to inactivate NDE. The transformed phenotype would then be assessed over time.

However, to our surprise (and dismay) NDE did not transform Schwann cells. As shown in Figure 10, while both NICD and NDE induced expression of Hes5 and Hey1, only NICD-transduced cells displayed elevated levels of c-Myc and Cyclin D1. NDE expression did not repress expression of Sox10 (data not shown). To ensure that this was not due simply to low expression from the retrovirus, we flow sorted NDE cells into high expressers (Hi) and low expressers (Lo) based on GFP levels. The high expressers also did not show evidence of transformation (Fig. 10).

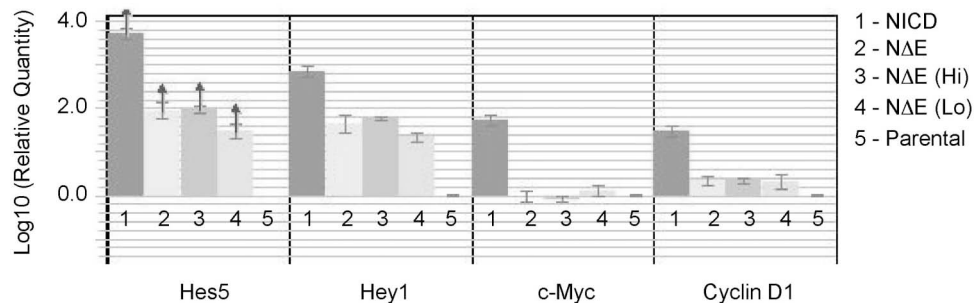


Figure 10. NDE does not transform Schwann cells. Cells expressing NDE (or sorted into high (Hi) or low (Lo) expressers) were evaluated for expression of the indicated genes by Q-RT-PCR and compared to parental Schwann cells and NICD-transformed Schwann cells.

NICD expression is NOT sufficient to transform rat Schwann cells. The results of Figure 10 forced us to re-examine our original observation that NICD is sufficient to transform Schwann cells. It should be stressed that our early experiences were very consistent and were reproduced many times: each time we transduced Schwann cells with a retrovirus expressing NICD we obtained transformed cells at high frequency. However, this has NOT been the case

over the past year. Using exactly the same retrovirus, we do not consistently observe transformed cells. The question is why?

It is known that simply growing primary cells in serum can lead to chromosomal rearrangements and transformation, particularly when the cells are “primed” with a known oncogene(10). Accordingly, we considered two possibilities. First, we explored the idea that the Schwann cells become transformed only after harboring NICD and grown in serum for extended periods of time. Second, we asked if the age of the cells at the time of transduction plays a role, with older cells being more susceptible. Our original experiments were carried out with cells that had been passed 30 times (which is fairly old). Our experiments did not support either of these ideas. As shown in Figure 11, using cells that had been transduced and immediately frozen three years ago, evidence of transformation (Sox10 down regulation) was apparent as soon as there was sufficient material to evaluate (passage 5). As shown in Figure 12, cells passed 30 times were not susceptible to transformation by NICD, unless the transformation had been carried out in 2004.

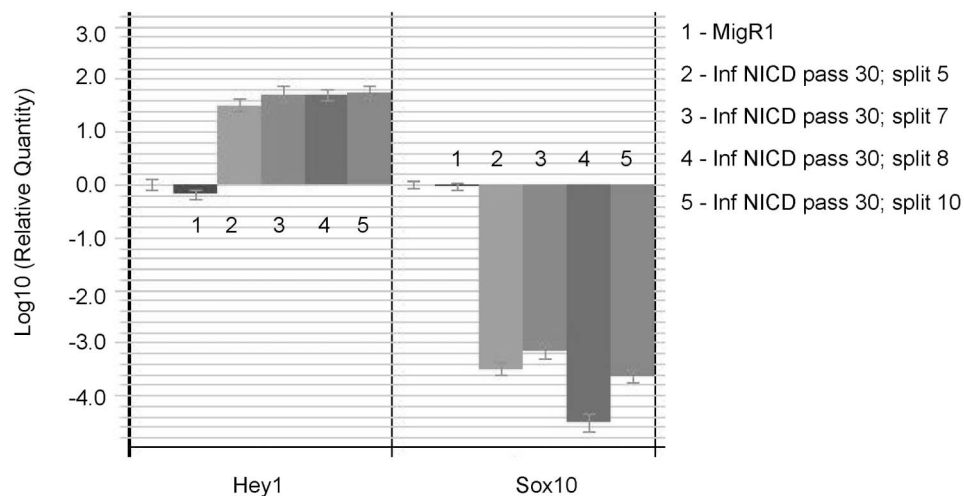


Figure 11. Cells transduced with NICD in 2004 show early evidence of transformation. Passage 30 Schwann cells transduced with NICD and immediately frozen in 2004 were thawed and passed (split) the indicated number of times and assessed for Hey1 expression and Sox 10 expression as indicated by Q-RT-PCR.

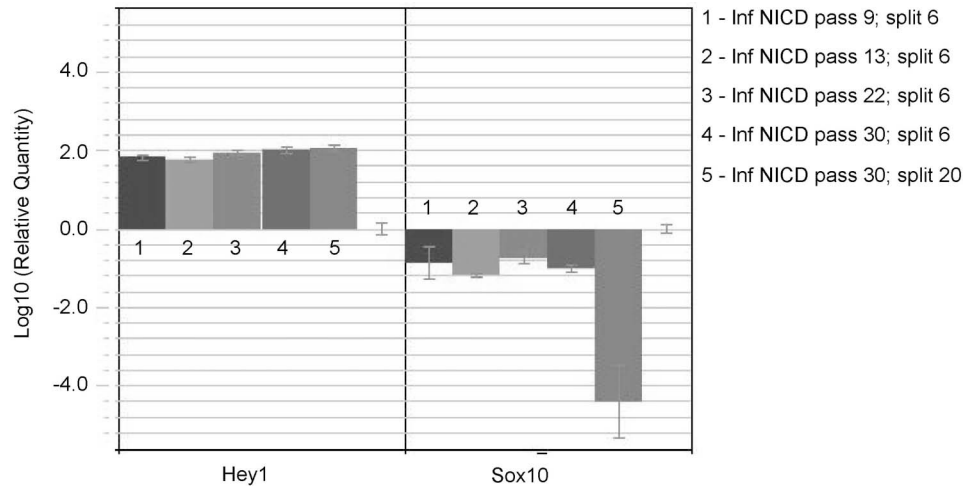


Figure 12. Old age does not guarantee transformation by NICD. Primary Schwann cells were passed for various times prior to transduction by an NICD-expressing virus. Populations 1 (passage 9), 2 (passage 13), 3 (passage 22) and 4 (passage 30) were transduced in 2006 and analyzed after 6 splits, while population 5 (passage 30) was transduced in 2004 and analyzed after 20 splits.

We conclude that under the conditions used in 2003-2004, NICD was capable of transforming Schwann cells, but those conditions do not exist today and remain elusive. Our current theory is that a particular batch of serum used in 2003-2004, which is no longer available, cooperated with NICD to transform the cells. As mentioned above, there is precedent for this possibility (10).

New Task: Determine which class of oncogenes cooperates with NICD to transform Schwann cells.

Dr. Allison Lloyd and colleagues have described a general model for the transformation of rat Schwann cells (11). Experiments employing activated Ras and various versions of SV40 Large T antigen showed that mitogen-independent growth can be distinguished from mitogen-independent *plus* anchorage-independent growth. Both are required for Schwann cells to be tumorigenic: the former required both activation of Ras and inactivation of p53, while the latter required, in addition, the inactivation of Rb family members, most likely p107 and/or p130. Interestingly, the ability of T antigen to repress Rb family proteins could be phenocopied by mutations in the cyclin-dependent kinase inhibitor p16^{Ink4a}. This suggests that p16^{Ink4a} mutations, while broadly stimulating CDK4 and CDK6, affect primarily the activities of p107 and p130, and not Rb. The reason for this is unknown.

We tested the hypothesis that NICD requires additional events to transform Schwann cells (as determined by anchorage independence) and that these will reflect one or a combination of those events described by Lloyd. Accordingly, we generated Schwann cells that were transduced with NICD plus either a) activated Ras, or b) SV40 Large T antigen. We anticipated that Ras plus T antigen would transform Schwann cells in the absence of NICD (5). According to our hypothesis, we expected NICD to fulfill the role of either activated Ras or T antigen.

An evaluation of cell growth in soft agar (a measure of anchorage independence) confirmed that

a combination of Ras plus T antigen led to robust colony formation (below right), while cells containing a) Ras alone (not shown), b) T antigen alone (not shown) or c) parental vectors alone (below left) produced no colonies as expected.



pBabe + LXS

T Antigen + Ras

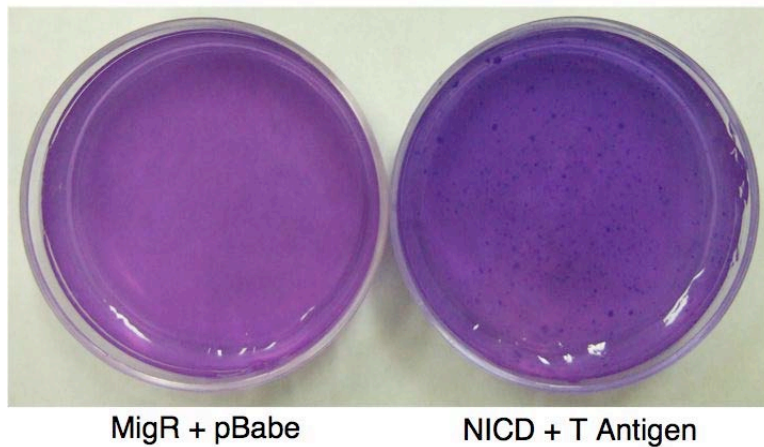
As reported previously, our initial experiments with NICD gave rise to modest colony formation in soft agar (below right) while cells expressing parental vector alone did not produce any growth (below left). Note, however, that the number of colonies was greatly reduced compared to the combination of Ras plus T antigen (8 vs. >400).



MigR

NICD

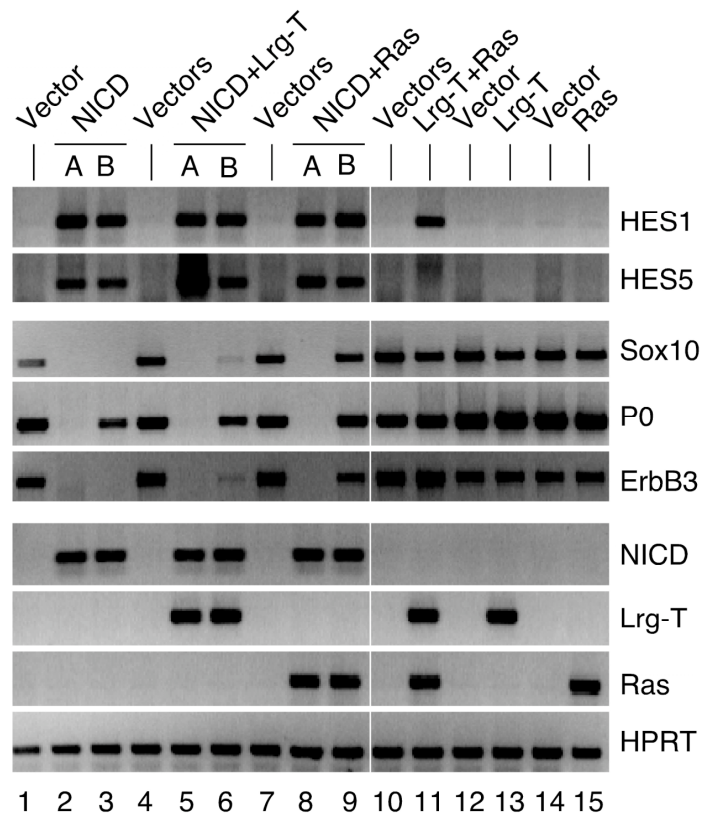
Importantly, a combination of NICD plus T antigen also gave robust growth in soft agar (below right, approx. 200 colonies), while NICD plus Ras did not increase the number of colonies over NICD alone (data not shown). *We conclude that NICD is fulfilling the role of Ras in promoting anchorage independent growth of Schwann cells.*



Is there a molecular signature for anchorage independence?

We had initially speculated that transformation of Schwann cells was accompanied by de-differentiation; namely, the loss of certain Schwann cell markers such as the transcription factor Sox10 and the myelin protein P0. In this case, the down-regulation of such markers by NICD would not be direct, but be secondary to the transformation process.

Given a better sense of what's required for transformation (i.e. anchorage independent growth), we have begun to explore this idea more rigorously. We have looked at gene expression by RT-PCR in Schwann cells transformed with various combinations of NICD, Ras and T-antigen. The results are shown below. Cells labeled "A" are the NICD-transduced cells we generated in our original studies. Cells labeled "B" are those generated within the past 6 months. "Lrg-T" indicates those cells transduced with a virus expressing SV40 Large T antigen. "Vectors" indicates those cells transduced with the relevant parental retroviruses.



Two conclusions can be drawn from the gene expression data shown above.

First, cell lines A and B (lanes 2 and 3) express similar levels of NICD and show similar induction of the Notch targets Hes1 and Hes 5. They also show similar down-regulation of Sox10, a likely Hes5 target. ErbB3 down-regulation is also similar in the two cell lines. However, down-regulation of P0, a Sox10 target, is much more robust in cell line A. The reason for this discrepancy is unknown.

Second, de-differentiation does not correlate with transformation. The cells capable of growing in soft agar are those transformed with combinations of Ras plus T antigen and of NICD plus T antigen (lanes 5, 6 and 11). Only those containing NICD showed down-regulation of Sox10 and ErbB3. Accordingly, it is now more likely that Sox10 and ErbB3 respond directly to Notch or to one of Notch's primary targets such as Hes1 or Hes5.

KEY RESEARCH ACCOMPLISHMENTS

1. Transformation of rat Schwann cells is due to a loss of contact inhibition.
2. Notch signaling down-regulates CyclinD1 expression in most cell types, including rat Schwann cells. Transformation, however, is accompanied by abundant CyclinD1 expression, suggesting a requirement for additional events in transformation.
3. Human MPNSTs, including those that express NICD, display reductions in CyclinD1 as a consequence of increased Notch signaling. This suggests that these cells are not as fully transformed as NICD-transduced rat Schwann cells.

4. NICD-transformed Schwann cells show an inherent increase in CyclinD1 levels and this correlates with an altered cell cycle profile.
5. Growth of NICD-transformed Schwann cells in soft agar requires signaling pathways emanating from PI3 Kinase and PKA.
6. Transcription of CyclinD1 is not affected by ligand-induced Notch signaling in normal Schwann cells and is increased roughly 2.5-fold in NICD-transformed Schwann cells.
7. The level of Sox10 transcripts is dramatically reduced (>1,000-fold) in NICD-transformed Schwann cells, suggesting a novel and important mode of Sox10 transcriptional regulation.
8. Neither Hes5 nor c-Myc, each a target of Notch, is able to mimic the ability of NICD to transform Schwann cells.
9. NICD expression is NOT sufficient to transform Schwann cells as previously thought.
10. NICD cooperates with SV40 Large T antigen to stimulate growth of Schwann cells in soft agar. Thus, NICD possesses activities similar to the Ras oncogene.
11. NICD-mediated down-regulation of Sox10 and ErbB3 is not secondary to Schwann cell transformation.

REPORTABLE OUTCOMES

None yet.

CONCLUSIONS

Perhaps the most important conclusion derived from our work is that human MPNST cells lines are not as fully transformed as NICD-transduced rat Schwann cells. Mouse modeling has shown that although Schwann cells are the primary targets of transformation, the micro-environment is also important, as genetic lesions in the Schwann cells alone is not sufficient to generate tumors. Accordingly, absolute proof for a role for Notch signaling in the development of MPNSTs may require mouse modeling.

Our data have refined the relationship between CyclinD1, the cell cycle and the growth rate of NICD-transformed Schwann cells. Solving an apparent paradox, we showed that increased CyclinD1 expression correlates with an increased S-phase population, but that an extended G2 phase may slow the cells growth relative to what we had expected. Our data are also consistent with the increase in CyclinD1 expression being due, in part, to transcriptional effects, possibly mediated by NICD directly.

Our data also show that normal levels of Notch signaling *are not sufficient* to mediate *any* of the observed effects of NICD. Thus, while Notch signaling may promote gliogenesis during development, transformation of Schwann cells appears largely unrelated and, in fact, correlates with de-differentiation.

Relationship between Notch and signaling through PI3 Kinase and PKA appear to be important for transformation, but such a relationship between Notch and Stat transcription factors, despite supporting evidence in neurons, does not appear to exist in glial cells.

While our data describing NICD-transformed Schwann cells remain valid, we can no longer conclude that NICD is *sufficient* for oncogenic transformation. We have now begun to unravel the mechanism through which NICD transforms Schwann cells and also down-regulates Sox10

and ErbB3. NICD appears to act similarly to Ras in promoting anchorage-independent growth. Transformation and Sox10/ErbB3 down-regulation are not necessarily linked.

Our initial hypothesis was that aberrant Notch signaling contributes to the development of MPNSTs. Our results argue against this hypothesis (note paragraph above) and suggest that Notch's role in Schwann cell transformation may be complex, requiring the collaboration of additional oncogenes. We still do not understand why our first experiments (those that served as the basis of this grant) so clearly demonstrated that NICD was sufficient for the transformation process.

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PERSONNEL FUNDED BY GRANT

Tom Kadesch
Ruth McCarrick-Walmsley
Jaojuan Liu

APPENDICES

None.